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Engineering microbial fatty acid metabolism for biofuels and biochemicals

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Traditional oleochemical industry chemically processes animal fats and plant oils to produce detergents, lubricants, biodiesel, plastics, coatings, and other products. Biotechnology offers an alternative process, where the same oleochemicals can be produced from abundant biomass feedstocks using microbial catalysis. This review summarizes the recent advances in the engineering of microbial metabolism for production of fatty acid-derived products. We highlight the efforts in engineering the central carbon metabolism, redox metabolism, controlling the chain length of the products, and obtaining metabolites with different functionalities. The prospects of commercializing microbial oleochemicals are also discussed.

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Introduction

Oleochemicals are a large group of fatty-acid derived compounds with an unprecedented application range: biodiesel, detergents, soaps, personal care products, industrial lubricants, plastic enhancers, bioplastics, emulsifiers, coatings, food and feed additives, and others [1]. Oleochemicals have traditionally been derived from vegetable oils and animal fats via chemical or enzymatic processes [2,3]. However, the limited availability, sustainability, and high cost of feedstocks limit the growth of this sector [4]. The long-term solution for this problem is sought in the expansion of feedstock range to more

abundant lignocellulosic biomass [5] and its conversion via chemical and microbial.

Using a microbial chassis in comparison to the traditional conversion of plant oils and animal fats presents a number of advantages. Firstly, feedstock availability is expanded from edible plant oils and animal fats to abundant first-generation and second-generation biomass feedstocks. Secondly, the feedstock-product dependence is eliminated as the desired oleochemicals can be obtained directly using an engineered cell factory from any feedstock, that is, a feedstock can be chosen based on the market price and availability. Finally, complex oleochemicals that cannot be obtained from natural sources because of low abundance can be produced by introducing novel synthetic biochemical pathways into platform chassis.

Microbial chassis must be extensively engineered in order to produce oleochemicals at high titer, rate, and yield for commercial exploitation [6]. The supply of metabolic precursors, acyl-CoAs, and redox co-factor NADPH need to be boosted (Figure 1). The chain length of the products needs to be controlled to obtain the required properties. One must also implement heterologous enzymes that will functionalize fatty acyl-CoAs into final products: hydroxylated and desaturated fatty acids, fatty alcohols, hydrocarbons, waxes, lactones, and others.

This review highlights the recent advances in the engineering of microbial metabolism towards the optimized production of natural and synthetic fatty-acid derived products. The microbial hosts covered in this review include common industrial workhorses: the bacterium *Escherichia coli*, the yeast *Saccharomyces cerevisiae*, and the oleaginous yeast *Yarrowia lipolytica*.

Engineering the central carbon metabolism

A common starting point for engineering microbial hosts is increasing the supply of fatty acid metabolic precursors: acetyl-CoA, malonyl-CoA, and fatty acyl-CoAs. In *E. coli*, it is effective to limit the fermentative pathways towards lactate, acetate, succinate, and ethanol, which consume acetyl-CoA. To circumvent the negative effects of these deletions on the cellular metabolism, these pathways can be first downregulated in the production phase, for example, Wu *et al.* applied CRISPR-based interference for repression of fermentative pathways and achieved 36% increase of the medium-chain fatty acid (MCFA) titer [7] (Table 1).

Table 1

Metabolic engineering strategies for fatty acid-derived metabolites.

Goals and genetic modifications ^a	Achievements	Host	Ref.
Improving acetyl-CoA supply			
Inducible $\downarrow adhE$, $\downarrow pta$, $\downarrow poxB$, $\downarrow dhA$, $\downarrow frdA$	1.36x C6-C10 FFA titer	Ec	[7]
Acetate recycling ($\uparrow acs$)	3.71x intracellular AcCoA level	Ec	[7]
$\uparrow PCC$ $\uparrow MCC$	2x FFA	Ec	[17]
$\uparrow ADH2$, $\uparrow ALD6$, $\uparrow acs^{FR}$	1.6–1.8x FAEE titer	Sc	[9]
$\uparrow YIACl$, $\downarrow MLS1$, $\Delta gpd1$	1.67x FFA titer	Sc	[11]
ACL route ($\uparrow MmusACL$ $\uparrow RtME$ $\uparrow MDH3'$ $\uparrow CTP1$)	1.18x FFA titer	Sc	[10*]
PDH-bypass ($\uparrow ScPDC1$ $\uparrow EcAldH$)	1.57x lipid titer	YI	[12**]
Pyruvate-formate lyase route ($\uparrow EcPflA$ $\uparrow EcPflB$)	1.47x lipid titer	YI	[12**]
Carnitine shuttle ($\uparrow ScCAT2$)	1.75x lipid titer	YI	[12**]
Non-oxidative PPP ($\uparrow AnPK$ $\uparrow BsPta$)	1.62x lipid titer	YI	[12**]
Increasing acetyl-CoA carboxylase flux			
Minimize acyl-CoA pool ($\Delta faa1$ $\Delta faa4$ $\uparrow MmusTes$)	4x ACC transcript level	Sc	[13]
$\uparrow ACB1$ $\uparrow ACC1^{FR}$	1.2–1.3x FAEE titers	Sc	[9]
$\uparrow OLE1$ $\uparrow ACC1$ $\uparrow DGA1$	3x lipid yield, 84.7% TM	YI	[14*]
Chain-length control			
RBO ($\uparrow RtBktB$ $\uparrow fadB$ $\uparrow EgTER$ $\uparrow ydiI$) $\Delta fadA$ $\Delta tesB$	1.1 g/L C6–C10 FFA (SF)	Ec	[29]
RBO ($\uparrow RtBktB$ $\uparrow fadB$ $\uparrow EgTER$ $\uparrow ydiI$) $\uparrow acs$ CRISPRi	3.8 g/L C6–C10 FFA (SF)	Ec	[7]
RBO ($\uparrow cytFOX3$ $\uparrow YIKR$ $\uparrow YIHTD$ $\uparrow cytoETR1$ $\uparrow EcEutE$) CaBdhB	12 mg/L C6–C10 FFA (SF)	Sc	[30]
FASi replacement ($\uparrow EcFAS$ $\Delta fas2$ $\uparrow RcFatB$)	~54 mg/L C14 (SF)	Sc	[27]
MPT swapping with Tes	380 mg/L C14 (SF)	YI	[12**]
FAS mutagenesis	118 mg/L C6–C8 FFA (SF)	Sc	[23]
Chimeric <i>RtFAS</i> -AcTes ($\uparrow cRtFAS$ -AbTes)	1.4 mg/L C6–C8 FFA, 0.3 mg/L C10–C12 FFA (SF)	Sc	[22*]
Product diversification^b			
Fatty alcohols (<i>MmarCAR</i> <i>BsSfp</i> <i>EcAHR</i> <i>EcFadD</i>)	2.15 g/L fatty alcohols (FB)	YI	[12**]
Alkanes ($\uparrow SeFAR$ $\uparrow SeADO$ $\uparrow SeFd/FNR$)	1.31 g/L alkanes (FB)	Ec	[46*]
Alkenes ($\uparrow JeOleT$ $\uparrow HEM3$ $\Delta ctt3$ Δcta $\Delta ccp1$)	3.7 mg/L (FB)	Ec	[47]
FAEEs (ER-localized <i>AbAtfA</i>)	136 mg/L (SF)	YI	[12**]
Linoleic acid (<i>Fvd12</i>)	1.3 g/L (SF)	Rt	[51]
GLA ($\uparrow Mald6$)	71.6 mg/L (SF)	YI	[35]
EPA (\uparrow heterologous-C16/C18E, d12, d9, d8, d5, d17)	>25% DCW	YI	[36*,37]
VLC-WE ($\Delta elo3$ $\uparrow ELO2$ $\uparrow MaqFAR$ $\uparrow SciWS$ $\uparrow ACC1^{FR}$)	15 mg/L VLC-WE (SF)	Sc	[48]
CBL lipid (cocoa GPAT, LPAT, and DGAT)	Titer not mentioned	Sc	[38]
HFA ($\Delta fadD$ $\uparrow ACC$ $\uparrow Tes$ $\uparrow BmCYP102Al$)	58.7 mg/L HFA (SF)	Ec	[40]
Methyl ketones ($\uparrow RtFAS$ - <i>ShMks2</i> $\uparrow ShMks1$)	10 μ g/gDCW (SF)	Sc	[22*]
Diacids (RBO, $\uparrow PpAlkBGT$ $\uparrow AcChnD$ $\uparrow AcChnE$)	0.5 g/L C6–C10 diacids (SF)	Ec	[29]
Docosanol ($\Delta elo3$ $\uparrow ELO1,2$ $\uparrow ACC1^{FR}$ $\uparrow AtFAR$ $\uparrow MvFAS$)	83.5 mg/L (SF)	Sc	[24]
Compartmentalization			
Per- <i>AbAtfA</i> or ER- <i>AbAtfA</i>	15x- (ER) to 19x- (Per) FAEE titer	YI	[12**]
ER- <i>AbFAR</i> and ER- <i>PmADO</i>	5.3x alkane titer	YI	[12**]
Per- <i>MaqFAR</i>	2.2x fatty alcohol titer	YI	[12**]
Per- <i>MmarCAR</i> , Per- <i>SeADO</i> , and Per- <i>SeFd/FNR</i>	1.9x alkanes titer	Sc	[31*]
Redox and cofactor engineering			
Increasing NADPH supply ($\uparrow CaGapC$ $\uparrow McMCE2$)	99 g/L lipid titer (FB, 98% TM)	YI	[19**]
The use of compatible Fd/FNR for ADO	1.7x alkane titer	Ec	[46*]
<i>JeOleT</i> cofactor supply ($\uparrow HEM3$ $\Delta ctt3$ Δcta Δccp)	1.2x alkene titer	Sc	[47]

The listed examples report the highest titers for corresponding products within the reviewed publications. **Symbols and prefixes** — ‘ \uparrow ’: over-expression; ‘ \downarrow ’: downregulation; ‘ Δ ’: deletion; cyto: cytosol-localized; ‘ $''$ ’: truncated version. **General abbreviations** — CRISPRi: CRISPR-based interference; SF: shake-flask; FB: fed-batch bioreactor; DCW: dry-cell weight. **Species abbreviations** — *Ab*: *Acinetobacter baylyi*; *Ac*: *Acinetobacter* sp.; *An*: *Aspergillus nidulans*; *Bm*: *Bacillus megaterium*; *Bs*: *Bacillus subtilis*; *Ca*: *Clostridium acetobutylicum*; *Ec*: *E. coli*; *Eg*: *Euglena gracilis*; *Fv*: *Fusarium verticillioides*; *Je*: *Jeotgalicoccus* sp.; *Mal*: *Mortierella alpina*; *Maq*: *Marinobacter aquaeolei*; *Mc*: *Mucor circinelloides*; *Mmar*: *Mycobacterium marinum*; *Mmus*: *Mus musculus*; *Mv*: *Mycobacterium vaccae*; *Pm*: *Prochlorococcus marinus*; *Pp*: *Pseudomonas putida*; *Rc*: *Ricinus communis*; *Rt*: *R. toruloides*; *Sci*: *Simmondsia chinensis*; *Se*: *Synechococcus elongatus*; *Sh*: *Solanum habrochaites*; *YI*: *Y. lipolytica*. **Gene/Enzyme abbreviations** — ACC: acetyl-CoA carboxylase; ADO: fatty-aldehyde deformylating oxygenase; AHR: aldehyde reductase; CAR: carboxylic acid reductase; C16/C18E: elongase converting palmitate (C16) to stearate (C18); d5, d8, d9, d12, d17: $\Delta 5$ -desaturase, $\Delta 8$ -desaturase, $\Delta 9$ -desaturase, $\Delta 12$ -desaturase, $\Delta 17$ -desaturase, respectively; DGAT: diacylglycerol O-acyltransferase; FAR: fatty acyl-CoA- or fatty acyl-ACP reductase; FAS: fatty-acid synthase; Fd/FNR: ferredoxin and ferredoxin/NADP⁺ reductase; GPAT: glyceryl-3-phosphate acyltransferase; HTD: β -hydroxyacyl-CoA dehydratase; KR: β -ketoacyl-reductase; LPAT: lysophosphatidate acyltransferase; ME: malic enzyme; Sfp: phosphopantetheinyl transferase; TER: trans- β -enoyl-CoA reductase; Tes: thioesterase; WS: wax-ester synthase. **Product abbreviations** — FAEE: fatty-acid ethyl esters; FFA: free fatty acids; HFA: hydroxylated fatty-acids; PK: phosphoketolase; VLC-WE: very-long-chain wax ester.

^a Unless indicated by a two-letter prefix of the species' name, the genes are native versions.

^b Except for GLA and EPA, only examples using minimal medium were considered.

The yeast *S. cerevisiae* does not naturally produce cytosolic acetyl-CoA at high levels and, to overcome this limitation, strategies circumventing the native pyruvate dehydrogenase reaction have been developed previously [8,9]. Recent studies reported the implementation of cytosolic acetyl-CoA generation via heterologous ATP:citrate lyase (ACL). Zhou *et al.* [10[•]] optimized a synthetic chimeric citrate lyase pathway by combining the expression of ATP:citrate lyase from *Mus musculus*, malic enzyme from *Rhodospiridium toruloides*, and overexpression of native mitochondrial citrate transporter and malate dehydrogenase, thus obtaining a 20% increase in the free fatty acid titer in *S. cerevisiae*. A strain that was additionally engineered for decreased degradation and activation of fatty acids and overexpression of acetyl-CoA carboxylase and fatty acid synthase, produced 1 g/L and 10.4 g/L of free fatty acids in correspondingly shake flask and fed-batch cultivations, the highest titers reported to date. A similar strategy, though using the ATP:citrate lyase from *Y. lipolytica*, was applied by Ghosh *et al.* [11] and resulted in a small improvement of the free fatty acid titer by 5%. The authors further carried out ¹³C metabolic flux analysis and identified that a large flux of acetyl-CoA was channeled into malate via malate synthase (Mls1p) and that additional carbon was lost to glycerol via glycerol-3-phosphate dehydrogenase (Gpd1p). Downregulation of *MLS1* and deletion of *GPD1* in addition to *ACL* overexpression, resulted in a 70% improvement over the reference strain, achieving ~0.8 g/L of free fatty acids in shake flasks.

The oleaginous yeast *Y. lipolytica* has a natural capacity of high-level production of cytosolic acetyl-CoA, particularly under nitrogen limitation when storage lipids are accumulated. To enable enhanced biosynthesis of oleochemicals already in the growth phase and to shorten the fermentation time, Xu *et al.* overexpressed several alternative routes towards acetyl-CoA formation [12^{••}]. As expected, overexpression of native *ACL* resulted in only a minor increase in lipid titer, but most of other strategies led to a significant improvement. Particularly, overexpression of carnitine acetyltransferase for enhanced export of mitochondrial acetyl-CoA into the cytosol led to a 75% improvement of the lipid titer. Moreover, the engineered strain began accumulating lipids already in the exponential growth phase before nitrogen became limiting. A final lipids titer of 66.4 g/L with an overall yield of 0.229 g/g glucose was achieved in a fed-batch cultivation.

Another important control point to increase the supply of malonyl-CoA is acetyl-CoA carboxylase (ACC), which is allosterically inhibited by C16-C20 saturated acyl-CoAs. This inhibition can be alleviated by deletion of fatty acid-CoA ligases (e.g., *FAA1* and *FAA4* in *S. cerevisiae*), which activate free fatty acids to acyl-CoAs [13]. Such a strategy is however not viable for production of acyl-CoA-derived metabolites and here an alternative approach was used by

Qiao *et al.* [14[•]]. They overexpressed stearyl-CoA desaturase in lipid-producing *Y. lipolytica* to decrease the concentration of stearyl-CoA, the main inhibitor of ACC. This resulted in a 3-fold increase of the lipid yield, reaching 84.7% of the theoretical maximum without growth impairment [14[•]]. Furthermore, through comparative genomic and transcriptomic analysis of lipid-overproducing strains, Liu *et al.* [15] identified a mutant Mga2p regulator, which leads to increased unsaturated fatty acid biosynthesis and lipid accumulation. Yet another approach is to apply dynamic regulatory modules to balance the precursor and product biosynthesis and avoid inhibition. A 2.1-fold improvement of the free fatty acid titer was obtained in *E. coli* with a regulatory circuit responding to malonyl-CoA concentration by upregulating either synthesis or consumption [16]. Shin *et al.* bypassed the ACC pathway by expressing methylmalonyl-CoA carboxyltransferase and phosphoenolpyruvate carboxylase, thus increasing the FFA titer about two-fold [17].

Engineering the redox metabolism

Fatty acid biosynthesis demands NADPH as a reducing co-factor, for example, 14 NADPH molecules are needed to produce one molecule of palmitoyl-CoA from acetyl-CoA. In *Y. lipolytica*, ¹³C metabolic flux analysis identified the oxidative pentose phosphate pathway as the primary source of NADPH for lipid biosynthesis [18]. However, generation of 2 NADPH through the pentose phosphate pathway costs one carbon, affecting the maximum theoretical yield. To improve the stoichiometry, Qiao *et al.* [19^{••}] tested several alternative strategies for NADPH generation in *Y. lipolytica*. The best-performing strain featured expression of NADP⁺-dependent glyceraldehyde 3-phosphate dehydrogenase from *Clostridium acetobutylicum* and of malic enzyme from *Mucor circinelloides*. The strain produced 99 g/L lipids with 1.2 g/L/h productivity, which to date, is the best production performance of a lipid-accumulating microbial strain.

Combining increased NADPH production with upregulation of oxidative stress defense pathways, improved the lipid yield by 82% and increased productivity by 5-fold. This strategy also changed the cellular morphology from pseudohyphae to single cells, which is beneficial for a large-scale fermentation process [20].

Controlling the chain length of products

Chain length is important for product properties, for example, gasoline-like and diesel-like biofuels require short/medium-chain fatty acids (S/MCFAs), waxes require very long chain (VLC) fatty acids and alcohols. Some specialty products such as lactone fragrances and polyunsaturated fatty acids require a precise chain length. Several methods to control the chain length of fatty acid products have been reported, including

engineering of fatty acid synthases, reverse beta-oxidation, and compartmentalization.

Type I fatty acid synthases

Fungal type I fatty acid synthases (FAS I) are large multi-modular proteins, releasing primarily palmitoyl-CoA. Due to the compact structure of fungal FAS I, the shorter acyl-CoA intermediates remain trapped in the enzyme complex and are not accessible for the cytosolic thioesterase. Various heterologous thioesterases have been overexpressed in *Y. lipolytica* and *S. cerevisiae*, and some smaller-sized thioesterases had an influence on the fatty acyl-CoA profiles [21,22[•]]. To obtain a better effect, Xu *et al.* replaced the malonyl/palmitoyl transferase domain (MPT) in *FAS1* of *Y. lipolytica* with heterologous thioesterases [12^{••}]. The resulting hybrid FAS I resulted in a three-fold increase of C14 acid, up to 29% of the total free fatty acids. Another approach was undertaken by Gajewski *et al.* [23]. They introduced mutations in the active sites of the β -ketoacyl synthase (KS), acetyl-CoA: ACP transacetyltransferase (AT), and malonyl/palmitoyl transferase (MPT) domains. The resulting mutated variants of FAS I produced up to ~ 0.5 g/L of extracellular short/medium-chain fatty acids (S/MCFA), predominantly hexanoate and octanoate, which do not occur in the wild type. Zhu *et al.* also created chimeras of fungal FAS I and thioesterases [22[•]]. *S. cerevisiae* strains expressing a chimera of FAS I of *R. toruloides* and a thioesterase of *Acinetobacter baylyi* produced up to 1.7 mg/L of short/medium chain fatty acids. This strategy was found superior to the expression of thioesterase in free form or as a C-terminal fusion to FAS I [22[•]]. The type I FAS from mycobacteria produces fatty acids of C16–C26 in length and has been used for the production of very long chain fatty acids in *S. cerevisiae* [24].

Type II fatty acid synthases

Bacteria possess a type II fatty acid synthase (FAS II), which is a protein complex assembled of multiple subunits. Through the expression of heterologous chain-specific thioesterases, the release of acyl-CoAs with a specific chain length can be achieved. This has been demonstrated in several studies. Jawed *et al.* identified a thioesterase from *Bryantella formatexigens* that enabled highly-specific butyric acid production using the native FAS in *E. coli* [25]. To enable the specific production of C7/9 alkanes, Sheppard *et al.* tested and optimized a thioesterase from *Cuphea hookeriana* and successfully produced octanoate and decanoate in *E. coli* without detecting other free fatty acids [26].

The success with manipulating the chain-length specificity of type II FAS in bacteria inspired the replacement of yeast FAS I with bacterial FAS II. In the study by Fernandez-Moya *et al.* [27], nine genes encoding enzymes in *E. coli* type II FAS were expressed in *S. cerevisiae*. The genes could partially compensate for the knock-out of

the native *FAS2*, albeit the growth was impaired. To alter the fatty acid chain-length profile, a thioesterase from *Ricinus communis* was used instead of *tesA*. This shifted chain-length profile of intracellular fatty acids from C18 to C14 fatty acids.

Reverse beta-oxidation

For each elongation cycle, which extends the acyl-CoA chain by two carbons, one malonyl-CoA (C3) is required and a carbon dioxide molecule is released. The biosynthesis of malonyl-CoA from acetyl-CoA requires an ATP and is hence energetically costly. As a more energy-efficient route than FAS [28], a reverse β -oxidation (RBO) pathway that uses acetyl-CoA instead of malonyl-CoA has been employed. Here, the initiation module, thiolase, and the last step, thioesterase, can be varied to control the chain length. Clomburg *et al.* [29] produced C6–C10 fatty acids by replacing native thiolase and thioesterase with the variants from *Ralstonia eutropha* [29]. Sheppard *et al.* successfully designed an *E. coli* strain that specifically produced pentane via reverse β -oxidation [26]. A reverse β -oxidation pathway has also been implemented in *S. cerevisiae* [30].

Compartmentalization

Xu *et al.* [12^{••}] showed that the fatty acid ethyl ester (FAEE) titer increased 10–15-fold when the esterifying enzyme was targeted to either the endoplasmic reticulum or the peroxisomes although a certain extent of product degradation was observed under peroxisomal localization. Localization of the alkane synthesis pathway into endoplasmic reticulum [12^{••}] or peroxisomes [12^{••},31[•]] also improved the alkane titer, partly because fatty aldehyde intermediates were isolated from the competing fatty alcohol biosynthesis [31[•]]. Sheng *et al.* [32] employed peroxisomal targeting of FAR to increase the production of medium chain-length fatty alcohols due to the higher abundance of acyl-CoA precursors in peroxisomes [32].

Functionalizing fatty acid intermediates into products

Lipids and specialty fatty acids

Oleaginous organisms can accumulate lipids at over 20% of the dry biomass and this capacity can be further increased by metabolic engineering. Oleaginous yeast *Y. lipolytica* was engineered by total to produce 85 g/L lipids with a productivity of 0.73 g/L/h. The relatively simple strategy comprised a careful selection of the parental strain, overexpression of heterologous diacylglycerol acyltransferases type 1 and 2, and deletion of the native lipase regulator *TGL3* [33].

The cells can also be engineered to change the profile of the acyl chains in lipids, for example, to produce lipids that contain hydroxylated or desaturated fatty acids. *Y. lipolytica* has been engineered to accumulate 12-hydroxy-9-cis-octadecenoic (ricinoleic) acid to 43% of total lipids

with a cellular content of 60 mg/g DW [34]. Ricinoleic acid is otherwise obtained from castor seeds, which presents a safety problem due to the ricin toxin. Polyunsaturated fatty acids (PUFAs) are attractive products due to their health and physiological benefits. Sun *et al.* engineered *Y. lipolytica* to accumulate up to 71.6 mg/L of γ -linolenic acid (an omega-6 acid) in lipids [35]. DuPont developed *Y. lipolytica* strains, capable of producing eicosapentaenoic acid (an omega-3 acid) at >25% DCW [36,37]. Manipulation of the TAG synthesis pathway to produce TAGs with specific combinations of acyl chains enabled the realization of cocoa-butter-like lipid accumulation in yeast [38,39].

Hydroxy fatty acids can also be produced in free form, for example, up to 548 mg/L of mixed hydroxylated acids were produced in *E. coli* engineered for improved fatty acid production and expressing the fatty acid hydroxylase (CYP102A1) from *Bacillus megaterium* [40].

Fatty alcohols

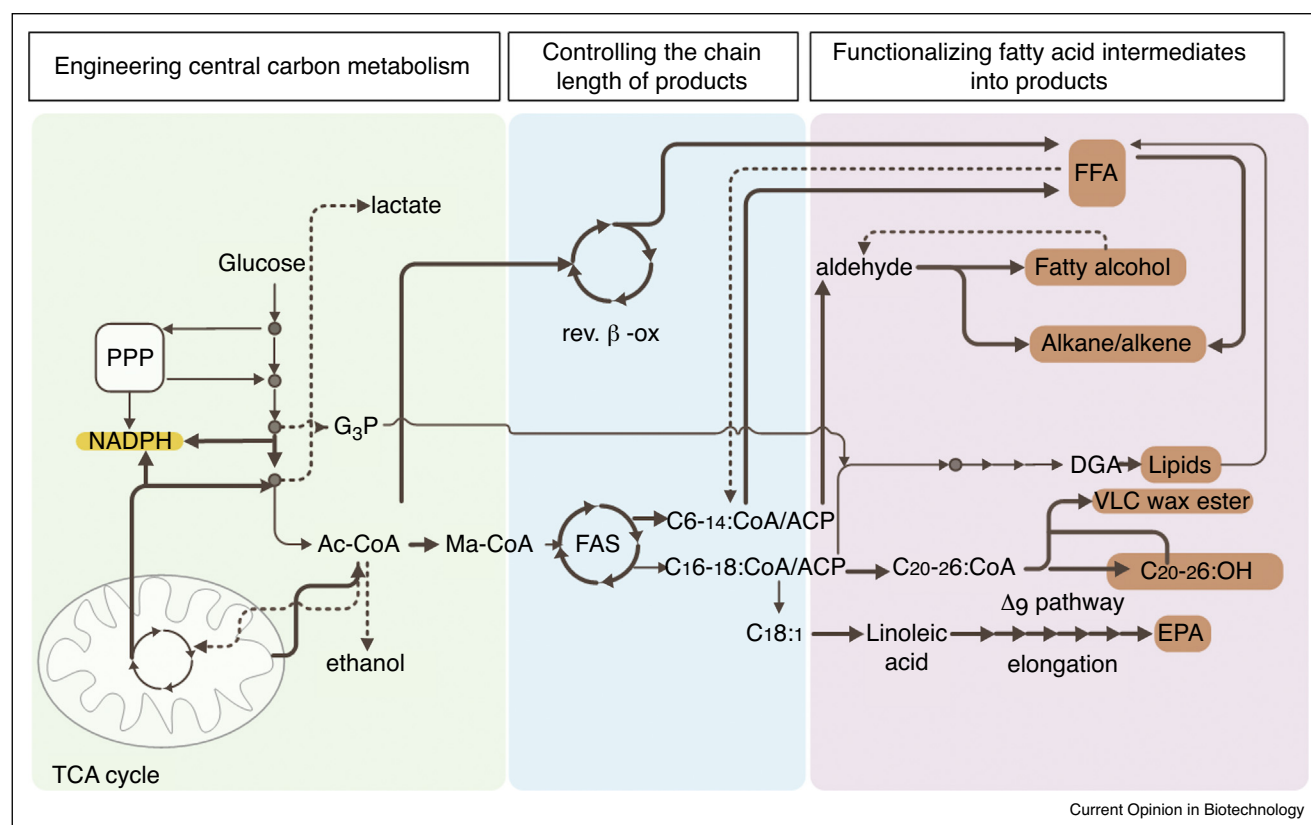
Fatty alcohols can be generated from fatty acids, fatty acyl-ACPs or from acyl-CoAs by fatty acid reductases,

which carry out the reduction either to aldehydes or all the way to alcohols. From fatty aldehydes, fatty alcohols can be produced relying solely on the endogenous activity of aldehyde reductase [31[•],32,41–43] although overexpressing either native [10[•]] or heterologous aldehyde reductases [12^{••}] has also been shown to enhance fatty alcohol production. Deletion of acyl-CoA synthases [10[•],44], deletion of acyl-ACP thioesterases [43], and overexpression of acyl-CoA synthases [12^{••}] were reported to improve fatty alcohol production when carboxylic acid reductase (CAR), acyl-ACP-specific fatty acyl reductase (FAR), and acyl-CoA-specific FAR were employed, respectively. Deletion of aldehyde reductase, catalyzing re-oxidation of fatty aldehydes into fatty acids, was shown to increase the production of fatty alcohols [31[•]].

Hydrocarbons

By combining fatty acyl reductases with aldehyde decarbonylases, alkanes can be obtained [45]. Cao *et al.* produced 1.3 g/L of alkanes in an engineered *E. coli* [46[•]]. In *S. cerevisiae*, peroxisomal localization of alkane biosynthesis improved the alkanes titer ca. 7-fold,

Figure 1



Strategies for tuning the microbial metabolism towards fatty acid-derived products. FFA: free fatty acids, VLC: very long chain, EPA: eicosapentaenoic acid.

resulting in 3.5 mg/L alkanes [31^{*}]. In *Y. lipolytica*, a 5-fold improvement was observed and the titer of alkanes was at 16.8 mg/L [12^{**}]. A proof-of-concept production of alkenes at 3.7 mg/L, mainly C17 and C19, was also demonstrated in *S. cerevisiae* with a heterologous cytochrome P450 fatty acid decarboxylase [47].

Waxes

Microbial synthesis of very-long-chain fatty acids (VLCFA) and their derivatives, with applications ranging from food to cosmetics [24], has been demonstrated in *S. cerevisiae* when using either endogenous long-chain fatty acid elongases or mycobacterial type I FAS [24,48]. Expression of FAR capable of using VLCFA led to the production of docosanol [24] and jojoba oil-like waxesters [48] (Figure 1).

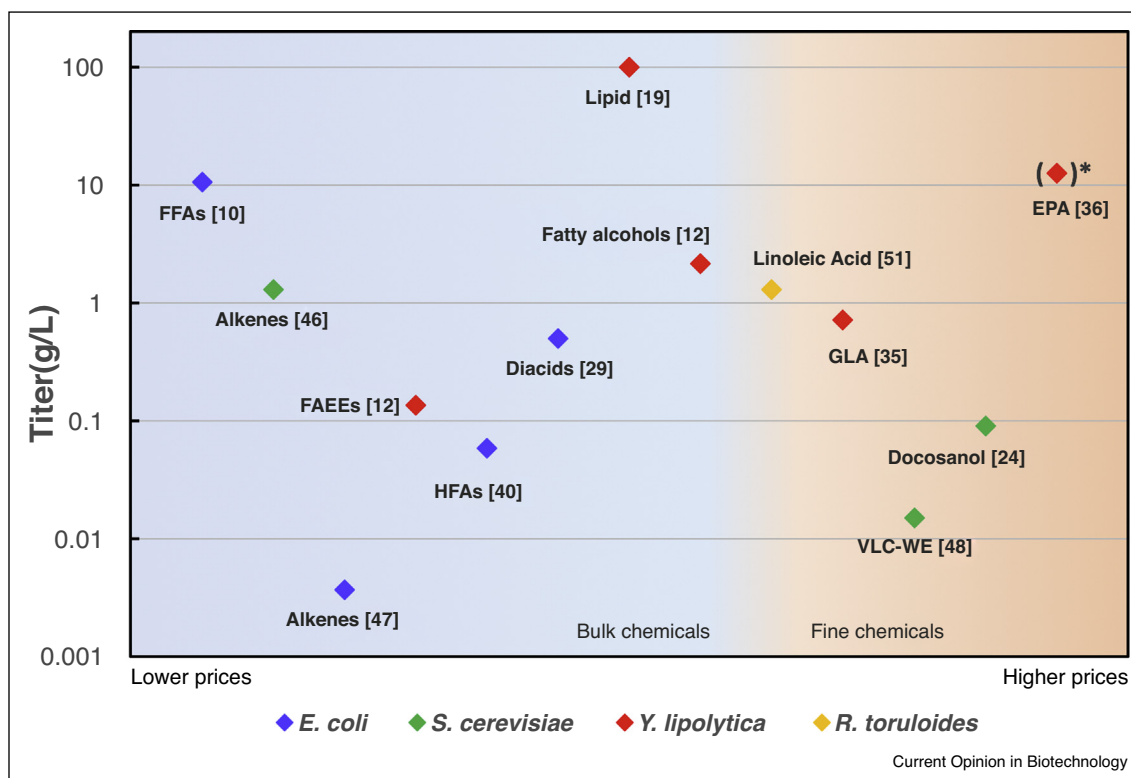
Perspectives

Oleochemicals produced by microbial fermentation have a prospect of contributing to the growth of the €18-billion global oleochemical market, and on a long run, they may also substitute many of the petrochemicals and fossil transportation fuels with a trillion-€-market. However, with the current favorable policies for the petrochemical

industry, the fermentation-based low-value oleochemicals would result in low profit margins even if the technology for their production were mature. From the technological maturity standpoint, the *Y. lipolytica*-based single-cell oil process developed by the Stephanopoulos group features the best production metrics, with a titer of 100 g/L and a nearly theoretical yield [19^{**}]. Additionally, engineered utilization of unnatural mineral sources may allow fermentation at non-sterile conditions [49]. Conversion of *Y. lipolytica* lipids with a high content of oleic acid into biodiesel will, however, require hydrogenation. Other microbial processes for the production of low-value oleochemicals, such as hydrocarbons, fatty alcohols, and esters, are still very far from commercialization point (Figure 2).

The commercial efforts in the next 5–10 years will likely focus on high-value specialty oleochemicals. Single cell oils enriched with polyunsaturated fatty acids (γ -linolenic, eicosapentaenoic, arachidonic, and docosahexaenoic acids) have been produced commercially since 90s using natural oleaginous algae and fungi [50], and recently also by engineered *Y. lipolytica* [36^{*}] and this market is expected to grow further. Examples of other attractive

Figure 2



Reported titers of fatty acid-derived products from microbial synthesis. FFAs: free fatty acids, References are shown in brackets. *EPA titer is approximated by assuming a cell dry-weight of 50 g/L.

high-value oleochemicals are specialty hydroxylated fatty acids for food and feed applications, waxes for cosmetics, lactone fragrances, and others.

Improving the performance metrics of the strains remains a major hurdle for the emergence of the new processes and continuous research on metabolic engineering strategies for improved oleochemicals production is a pre-request for success.

Conflict of interest

IB and CH are co-founders of BioPhero ApS. VS is co-founder of Biopetrolia.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Pfleger BF, Gossing M, Nielsen J: **Metabolic engineering strategies for microbial synthesis of oleochemicals.** *Metab Eng* 2015, **29**:1-11.
 2. Zhu L-H, Krens F, Smith MA, Li X, Qi W, Loo EN van, Iven T, Feussner I, Nazarenus TJ, Huai D et al.: **Dedicated industrial oilseed crops as metabolic engineering platforms for sustainable industrial feedstock production.** *Sci Rep* 2016, **6**: srep22181.
 3. Bhuiya MMK, Rasul MG, Khan MMK, Ashwath N, Azad AK: **Prospects of 2nd generation biodiesel as a sustainable fuel — Part: 1 selection of feedstocks, oil extraction techniques and conversion technologies.** *Renew Sustain Energy Rev* 2016, **55**:1109-1128.
 4. Schmidt JH: **Life cycle assessment of five vegetable oils.** *J Clean Prod* 2015, **87**:130-138.
 5. Isikgor H, Remzi Becer FC: **Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers.** *Polym Chem* 2015, **6**:4497-4559.
 6. Jin M, Slininger PJ, Dien BS, Waghmode S, Moser BR, Orjuela A, Sousa L, da C, Balan V: **Microbial lipid-based lignocellulosic biorefinery: feasibility and challenges.** *Trends Biotechnol* 2015, **33**:43-54.
 7. Wu J, Zhang X, Xia X, Dong M: **A systematic optimization of medium chain fatty acid biosynthesis via the reverse beta-oxidation cycle in *Escherichia coli*.** *Metab Eng* 2017, **41**:115-124.
 8. Krivoruchko A, Zhang Y, Siewers V, Chen Y, Nielsen J: **Microbial acetyl-CoA metabolism and metabolic engineering.** *Metab Eng* 2015, **28**:28-42.
 9. Jong BW de, Shi S, Siewers V, Nielsen J: **Improved production of fatty acid ethyl esters in *Saccharomyces cerevisiae* through up-regulation of the ethanol degradation pathway and expression of the heterologous phosphoketolase pathway.** *Microb Cell Factories* 2014, **13**:39.
 10. Zhou YJ, Buijs NA, Zhu Z, Qin J, Siewers V, Nielsen J: **Production of fatty acid-derived oleochemicals and biofuels by synthetic yeast cell factories.** *Nat Commun* 2016, **7**: ncomms11709.
- Achieved the highest so far free fatty acids titer (10 g/L) through optimization of cytosolic acetyl-CoA supply in *S. cerevisiae*. Investigated different combinations of expression of heterologous and native pathways as well as deletion of competing pathways for improved production of fatty alcohols and alkanes.
11. Ghosh A, Ando D, Gin J, Runguphan W, Denby C, Wang G, Baidoo EEK, Shymansky C, Keasling JD, Garcia Martin H: **13C metabolic flux analysis for systematic metabolic engineering of *S. cerevisiae* for overproduction of fatty acids.** *Front Bioeng Biotechnol* 2016:4.
 12. Xu P, Qiao K, Ahn WS, Stephanopoulos G: **Engineering *Yarrowia lipolytica* as a platform for synthesis of drop-in transportation fuels and oleochemicals.** *Proc Natl Acad Sci* 2016, **113**:10848-10853.
- First demonstration of fungal type I FAS engineering to obtain medium-chain-fatty acids. Presents various compartmentalization strategies, rewiring central carbon metabolism, and decoupling the product formation from growth.
13. Chen L, Zhang J, Lee J, Chen WN: **Enhancement of free fatty acid production in *Saccharomyces cerevisiae* by control of fatty acyl-CoA metabolism.** *Appl Microbiol Biotechnol* 2014, **98**:6739-6750.
 14. Qiao K, Imam Abidi SH, Liu H, Zhang H, Chakraborty S, Watson N, Kumaran Ajikumar P, Stephanopoulos G: **Engineering lipid overproduction in the oleaginous yeast *Yarrowia lipolytica*.** *Metab Eng* 2015, **29**:56-65.
- The study presents a novel, elegant approach to avoid the repression of acetyl-CoA carboxylase by increasing fatty-acyl-CoA desaturation. The result is a *Y. lipolytica* strain with improved growth, increased tolerance to sugars, and high-level lipid production.
15. Liu L, Markham K, Blazeck J, Zhou N, Leon D, Otoupal P, Alper HS: **Surveying the lipogenesis landscape in *Yarrowia lipolytica* through understanding the function of a Mga2p regulatory protein mutant.** *Metab Eng* 2015, **31**:102-111.
 16. Xu P, Li L, Zhang F, Stephanopoulos G, Koffas M: **Improving fatty acids production by engineering dynamic pathway regulation and metabolic control.** *Proc Natl Acad Sci* 2014, **111**:11299-11304.
 17. Shin KS, Lee SK: **Introduction of an acetyl-CoA carboxylation bypass into *Escherichia coli* for enhanced free fatty acid production.** *Bioresour Technol* 2017:169 <http://dx.doi.org/10.1016/j.biortech.2017.05>.
 18. Wasylenko TM, Ahn WS, Stephanopoulos G: **The oxidative pentose phosphate pathway is the primary source of NADPH for lipid overproduction from glucose in *Yarrowia lipolytica*.** *Metab Eng* 2015, **30**:27-39.
 19. Qiao K, Wasylenko TM, Zhou K, Xu P, Stephanopoulos G: **Lipid production in *Yarrowia lipolytica* is maximized by engineering cytosolic redox metabolism.** *Nat Biotechnol* 2017, **35**:173-177.
- This is the first demonstration that modulation of NADPH recovery pathways in *Y. lipolytica* can increase the theoretical maximum of lipid accumulation.
20. Xu P, Qiao K, Stephanopoulos G: **Engineering oxidative stress defense pathways to build a robust lipid production platform in *Yarrowia lipolytica*.** *Biotechnol Bioeng* 2017, **114**:1521-1530.
 21. Rutter CD, Zhang S, Rao CV: **Engineering *Yarrowia lipolytica* for production of medium-chain fatty acids.** *Appl Microbiol Biotechnol* 2015, **99**:7359-7368.
 22. Zhu Z, Zhou YJ, Krivoruchko A, Grininger M, Zhao ZK, Nielsen J: **Expanding the product portfolio of fungal type I fatty acid synthases.** *Nat Chem Biol* 2017, **13**:360-362.
- Introduction of heterologous domains into reaction chamber of *S. cerevisiae*'s fatty acid synthase enables production of short-chain and medium-chain fatty acids.
23. Gajewski J, Pavlovic R, Fischer M, Boles E, Grininger M: **Engineering fungal *de novo* fatty acid synthesis for short chain fatty acid production.** *Nat Commun* 2017, **8**: ncomms14650.
 24. Yu T, Zhou YJ, Wenning L, Liu Q, Krivoruchko A, Siewers V, Nielsen J, David F: **Metabolic engineering of *Saccharomyces cerevisiae* for production of very long chain fatty acid-derived chemicals.** *Nat Commun* 2017, **8**: ncomms15587.
 25. Jawed K, Mattam AJ, Fatma Z, Wajid S, Abdin MZ, Yazdani SS: **Engineered production of short chain fatty acid in *Escherichia coli* using fatty acid synthesis pathway.** *PLOS ONE* 2016, **11**: e0160035.

26. Sheppard MJ, Kunjapur AM, Prather KLJ: **Modular and selective biosynthesis of gasoline-range alkanes**. *Metab Eng* 2016, **33**:28-40.
 27. Fernandez-Moya R, Leber C, Cardenas J, Da Silva NA: **Functional replacement of the *Saccharomyces cerevisiae* fatty acid synthase with a bacterial type II system allows flexible product profiles**. *Biotechnol Bioeng* 2015, **112**:2618-2623.
 28. Kim S, Clomburg JM, Gonzalez R: **Synthesis of medium-chain length (C6–C10) fuels and chemicals via β -oxidation reversal in *Escherichia coli***. *J Ind Microbiol Biotechnol* 2015, **42**:465-475.
 29. Clomburg JM, Blankschien MD, Vick JE, Chou A, Kim S, Gonzalez R: **Integrated engineering of β -oxidation reversal and ω -oxidation pathways for the synthesis of medium chain ω -functionalized carboxylic acids**. *Metab Eng* 2015, **28**:202-212.
 30. Lian J, Zhao H: **Reversal of the β -oxidation cycle in *Saccharomyces cerevisiae* for production of fuels and chemicals**. *ACS Synth Biol* 2015, **4**:332-341.
 31. Zhou YJ, Buijs NA, Zhu Z, Gómez DO, Boonsombuti A, Siewers V, Nielsen J: **Harnessing yeast peroxisomes for biosynthesis of fatty-acid-derived biofuels and chemicals with relieved side-pathway competition**. *J Am Chem Soc* 2016, **138**:15368-15377.
- The authors show that localizing the pathway for alkane biosynthesis in peroxisomes significantly decreases the degradation of pathway intermediates, fatty aldehydes, and improves the overall alkane production.
32. Sheng J, Stevens J, Feng X: **Pathway compartmentalization in peroxisome of *Saccharomyces cerevisiae* to produce versatile medium chain fatty alcohols**. *Sci Rep* 2016, **6**:srep26884.
 33. Friedlander J, Tsakraklides V, Kamineni A, Greenhagen EH, Consiglio AL, MacEwen K, Crabtree DV, Afshar J, Nugent RL, Hamilton MA *et al.*: **Engineering of a high lipid producing *Yarrowia lipolytica* strain**. *Biotechnol Biofuels* 2016, **9**:77.
 34. Beopoulos A, Verbeke J, Bordes F, Guicherd M, Bressy M, Marty A, Nicaud J-M: **Metabolic engineering for ricinoleic acid production in the oleaginous yeast *Yarrowia lipolytica***. *Appl Microbiol Biotechnol* 2014, **98**:251-262.
 35. Sun M-L, Madzak C, Liu H-H, Song P, Ren L-J, Huang H, Ji X-J: **Engineering *Yarrowia lipolytica* for efficient γ -linolenic acid production**. *Biochem Eng J* 2017, **117**(Part A):172-180.
 36. Xie D, Jackson EN, Zhu Q: **Sustainable source of omega-3 eicosapentaenoic acid from metabolically engineered *Yarrowia lipolytica*: from fundamental research to commercial production**. *Appl Microbiol Biotechnol* 2015, **99**:1599-1610.
- Summarizes the process for developing *Y. lipolytica* strains for commercial production of an omega-3 acid.
37. Xue Z, Sharpe PL, Hong S-P, Yadav NS, Xie D, Short DR, Damude HG, Rupert RA, Seip JE, Wang J *et al.*: **Production of omega-3 eicosapentaenoic acid by metabolic engineering of *Yarrowia lipolytica***. *Nat Biotechnol* 2013, **31**:734-740.
 38. Wei Y, Gossing M, Bergenholm D, Siewers V, Nielsen J: **Increasing cocoa butter-like lipid production of *Saccharomyces cerevisiae* by expression of selected cocoa genes**. *AMB Express* 2017, **7**:34.
 39. Wei Y, Siewers V, Nielsen J: **Cocoa butter-like lipid production ability of non-oleaginous and oleaginous yeasts under nitrogen-limited culture conditions**. *Appl Microbiol Biotechnol* 2017, **101**:3577-3585.
 40. Cao Y, Cheng T, Zhao G, Niu W, Guo J, Xian M, Liu H: **Metabolic engineering of *Escherichia coli* for the production of hydroxy fatty acids from glucose**. *BMC Biotechnol* 2016, **16**:26.
 41. Buijs NA, Zhou YJ, Siewers V, Nielsen J: **Long-chain alkane production by the yeast *Saccharomyces cerevisiae***. *Biotechnol Bioeng* 2015, **112**:1275-1279.
 42. Rutter CD, Rao CV: **Production of 1-decanol by metabolically engineered *Yarrowia lipolytica***. *Metab Eng* 2016, **38**:139-147.
 43. Liu Y, Chen S, Chen J, Zhou J, Wang Y, Yang M, Qi X, Xing J, Wang Q, Ma Y: **High production of fatty alcohols in *Escherichia coli* with fatty acid starvation**. *Microb Cell Factories* 2016, **15**:129.
 44. Teixeira PG, Ferreira R, Zhou YJ, Siewers V, Nielsen J: **Dynamic regulation of fatty acid pools for improved production of fatty alcohols in *Saccharomyces cerevisiae***. *Microb Cell Factories* 2017, **16**:45.
 45. Kang M-K, Zhou YJ, Buijs NA, Nielsen J: **Functional screening of aldehyde decarbonylases for long-chain alkane production by *Saccharomyces cerevisiae***. *Microb Cell Factories* 2017, **16**:74.
 46. Cao Y-X, Xiao W-H, Zhang J-L, Xie Z-X, Ding M-Z, Yuan Y-J: **Heterologous biosynthesis and manipulation of alkanes in *Escherichia coli***. *Metab Eng* 2016, **38**:19-28.
- The study reports so far the highest titer of alkanes obtained in a microbial process. The authors systematically apply a variety of metabolic engineering strategies to optimize an *E. coli* strain.
47. Chen B, Lee D-Y, Chang MW: **Combinatorial metabolic engineering of *Saccharomyces cerevisiae* for terminal alkene production**. *Metab Eng* 2015, **31**:53-61.
 48. Wenning L, Yu T, David F, Nielsen J, Siewers V: **Establishing very long-chain fatty alcohol and wax ester biosynthesis in *Saccharomyces cerevisiae***. *Biotechnol Bioeng* 2017, **114**:1025-1035.
 49. Shaw AJ, Lam FH, Hamilton M, Consiglio A, MacEwen K, Brevnova EE, Greenhagen E, LaTouf WG, South CR, Dijken H van *et al.*: **Metabolic engineering of microbial competitive advantage for industrial fermentation processes**. *Science* 2016, **353**:583-586.
 50. Ratledge C: **Microbial oils: an introductory overview of current status and future prospects**. *OCL* 2013, **20**:D602.
 51. Wang Y, Zhang S, Pötter M, Sun W, Li L, Yang X, Jiao X, Zhao ZK: **Overexpression of $\Delta 12$ -fatty acid desaturase in the oleaginous yeast *Rhodospiridium toruloides* for production of linoleic acid-rich lipids**. *Appl Biochem Biotechnol* 2016, **180**:1497-1507.